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Differential sensitivity of mouse pronuclei and zygote cytoplasm to Hoechst staining and ultraviolet irradiation

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Summary. The exposure of mouse zygotes pre-stained with Hoechst 33342 to u.v. irradiation for 20–30 sec significantly or completely inhibited development to blastocysts *in vitro*. However, development to the blastocyst stage of enucleated eggs receiving pronuclei from untreated eggs was as good as that of control reconstituted eggs when the cytoplasm originated from eggs exposed to u.v. irradiation for 20–30 sec, but was significantly lower when the cytoplasm was from eggs exposed for 40 sec. The chromosomes at the second metaphase stage could be removed with 15 sec of exposure to u.v. irradiation under a fluorescence microscope. Most eggs enucleated at the second metaphase that received a single inner cell mass nucleus (75%) showed pronuclear formation 6 h after activation; 23% of them developed to morphologically normal 2-cell eggs and 5% developed to blastocysts.

These results demonstrate that the cytoplasm of mouse zygotes is more resistant to u.v. irradiation after Hoechst staining. Eggs at the second metaphase, from which chromosomes have been removed under a fluorescence microscope, can therefore be used as cytoplasm recipients for nuclear transplantation of inner cell mass nuclei.

Keywords: u.v. irradiation; Hoechst staining; nuclear transplantation; mouse egg

Introduction

One of the purposes of studying nuclear transplantation is to attempt to obtain offspring from nuclei of differentiated cells so that cloned offspring can be produced. Although we succeeded in producing offspring after transplantation of mouse 4- and 8-cell nuclei, no development was observed after transplantation of inner cell mass nuclei (Tsunoda *et al.*, 1987). One possible approach to the problem is to find a method of converting the nucleus into a 'fertilization' nucleus, as has been reported for the frog (Hoffner & DiBerardino, 1980). Nuclei from mouse follicle cells (Tarkowski & Balakier, 1980) or thymocytes (Czolowska *et al.*, 1984), when fused with oocytes at the second metaphase, show the nuclear swelling and decondensation of chromatin which are prerequisites for nuclear reprogramming (DiBerardino, 1980).

There are technical difficulties in observing and manipulating mouse chromosomes at metaphase under a phase-contrast or interference microscope. Chromosomes at metaphase can be easily recognized under a fluorescence microscope when they are stained with Hoechst dye (Ebert *et al.*, 1985; Tone & Kato, 1986). However, u.v. irradiation damages the developmental ability of preimplantation mouse eggs, especially in combination with Hoechst staining (Eibs & Spielmann, 1977; Ebert *et al.*, 1985).

The purpose of the present study was to examine (1) whether the sensitivity to u.v. irradiation and Hoechst staining is different for pronuclei and cytoplasm of mouse zygotes and, if so, (2)

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whether the enucleated cytoplasm subjected to u.v. irradiation and Hoechst staining can be used for nuclear transplantation.

Materials and Methods

Adult mice were superovulated with injections of 5 i.u. PMSG and 5 i.u. hCG 48 h apart and mated. Eggs at the pronuclear stage were obtained 20–24 h after hCG injection. Cumulus cells were removed by treatment with hyaluronidase (300 i.u./ml) in Medium M2 (Fulton & Whittingham, 1978) and washed three times. The eggs for different treatments and cytoplasm for recipient eggs (Series 1) were obtained from non-albino F1 (C57BL/6J × CBA) female mice mated to males of the same strain. The unfertilized eggs used for cytoplasm recipient (Series 2) were obtained from superovulated F1 females 15–17 h after hCG injection. Pronuclei (Series 1) or nuclei from inner cell mass cells (Series 2) were obtained from donor eggs of albino CD-1 females mated to males of the same strain.

After treatment or nuclear transplantation, the eggs were washed several times and cultured in individual drops of Medium M16 (Whittingham, 1971) with or without 100 μM-EDTA for 24 h to the 2-cell stage, and for 96 h to the blastocyst stage, under liquid paraffin in a 5% CO_2 and 95% air atmosphere at 37°C. Some eggs that developed to blastocysts were transferred to uteri of pseudopregnant females 2·5 days *post coitum* to examine the number of live fetuses.

Before nuclear transplantation, the zonae pellucidae of all the eggs were slit with a fine glass needle along 10–20% of their circumference. The methods for zona cutting, setting the holding and enucleation pipette, pretreatment of eggs with cytochalasin B and colcemid, and nuclear transplantation were as described by Tsunoda *et al.* (1986).

Series 1. In Exp. 1, the effect of staining with Hoechst dye on the developmental ability of mouse zygotes was examined. The eggs were incubated with Medium M16 containing 0·25–10 μg/ml Hoechst 33342 (Calbiochem-Behring Corp., San Diego, CA, U.S.A.; No. 382065) for 3 min at 37°C, washed several times and then cultured for 96 h. The working solution of Hoechst dye in Medium M16 was prepared daily from a stock solution (1 mg/ml in water) (Pursel *et al.*, 1985).

In Exp. 2, the zygotes preincubated with different concentrations of Hoechst dye (0–2·5 μg/ml) were placed in a small drop of Medium M2 on a micromanipulation glass slide covered with paraffin oil, and placed on the stage of a fluorescence microscope for 0–360 sec. The microscope was a Nikkon inverted microscope with a fluorescence condenser (blue violet excitation filter with 400–450 nm wave) and an Osram (HBO-100) superpressure mercury lamp. Although the fluorescence intensity was not measured, it was controlled throughout the study by using the same excitation filter, same magnification (× 200) and same lamp (100 W, 100–150 h after use).

In Exp. 3, male and female pronuclei obtained from fresh zygotes were introduced into the perivitelline space of enucleated zygotes previously exposed to u.v. light for 20–40 sec after staining with Hoechst dye (0·5 μg/ml) for 3 min.

Series 2. A single donor inner cell mass cell was obtained from a blastocyst 3·5 days *post coitum* by immunosurgery. The zona pellucida was removed by incubation with 0·5% pronase in Medium M2. The blastocyst was incubated with the IgG fraction of a rabbit antibody against mouse liver and kidney (1 mg/ml in Medium M2) following treatment with guinea-pig complement (1:7 v/v) for 30 min at 35°C. The isolated inner cell mass was incubated in Ca^{2+}, Mg^{2+}-free Medium PB1 (Whittingham & Wales, 1969) supplemented with 0·2% EDTA at 35°C for 45 min and then pipetted into single cells (Modlinski, 1981). The inner cell mass cells were stored at 4°C for 4 h in Medium PB1 supplemented with 20% FCS until nuclear transplantation. The zonae pellucidae of the eggs at the second metaphase stage were slit; the eggs were then stained with Hoechst 33342 (0·5 μg/ml) for 3 min, and incubated with Medium M2 containing cytochalasin B and colcemid for 30 min at room temperature. This concentration of Hoechst dye was used because this was the lowest concentration to show moderate fluorescence of metaphase chromosomes. The second metaphase chromosomes were removed with an enucleation pipette by using a micromanipulator under a fluorescence microscope; the egg was first secured by a holding pipette opposite the slit in the zona, and the enucleation pipette was inserted into the perivitelline space through the slit. The plate interrupting the u.v. rays was then removed (Fig. 1), the chromosomes were sucked into the enucleation pipette (Fig. 2), and the plate was replaced into the pathway of the u.v. rays. The actual time of exposure to u.v. irradiation was 6–15 sec (average 12 sec). Then an inner cell mass nucleus with inactivated Sendai virus (HVJ) (2700 haemagglutinating activity units/ml) was introduced into the perivitelline space of the enucleated egg. At 5–30 min after the injection, the eggs were activated with 7% ethanol for 7 min at room temperature (Cuthbertson, 1983).

Results

Series 1

Table 1 shows the results of in-vitro development of mouse zygotes stained with Hoechst 33342 at various concentrations. The proportions of eggs developed to blastocysts after staining with Hoechst dye at concentrations of 0·25–5 μg/ml (75–91%) were not significantly different from that obtained in the control group (88%). The development of eggs stained at concentrations of 7·5 and 10 μg/ml (38 and 12%) was significantly ($P < 0·001$) reduced compared with that in the control

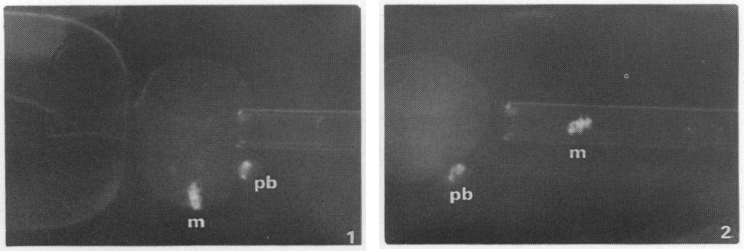

Fig. 1. An egg prestained with Hoechst dye (0·5 μg/ml) is secured by a holding pipette (left) and the enucleation pipette is inserted into the perivitelline space through the slit on the zona. The plate interrupting the u.v. ray was then removed. The second metaphase chromosomes (m) and the first polar body (pb) can be seen.

Fig. 2. The second metaphase chromosomes (m) have been sucked into the enucleation pipette. pb = first polar body.

Table 1. The effect of Hoechst 33342 dye on in-vitro* development of mouse zygotes

Conc. of dye (μg/ml)	No. of eggs used	No. of blastocysts (%)
0	75	66 (88)
0·25	61	55 (90)
0·5	59	52 (88)
1·0	53	48 (91)
2·5	56	51 (91)
5·0	52	39 (75)
7·5	50	19 (38)**
10·0	57	7 (12)**

*The eggs were incubated for 3 min at 37°C.
**$P < 0·001$.

group. Moderate (++) to strong (+++) fluorescence was observed on pronuclei of zygotes treated with Hoechst dye at concentrations of 0·5–10 μg/ml, but weak (+) fluorescence was observed after staining with Hoechst at a concentration of 0·25 μg/ml.

Table 2 shows in-vitro development of zygotes stained with Hoechst dye (0·5–2·5 μg/ml) after exposure to u.v. light under a fluorescence microscope for different times. The exposure to only u.v. light for 5–60 sec did not adversely affect the development of eggs to the blastocyst stage, but exposure for 120–360 sec significantly ($P < 0·001$) inhibited the development of eggs. However, the exposure to u.v. light had a dramatic effect on the number of blastocysts developed from zygotes following staining with Hoechst dye. Exposure for 5 sec significantly ($P < 0·001$) inhibited the development of eggs to blastocysts compared with that of control eggs, and exposure for longer than 30 sec completely inhibited development to the blastocyst stage. The inhibition of the development of eggs exposed to the dye and u.v. light increased as the dye concentration increased.

Table 3 shows the development of reconstituted eggs whose pronuclei were obtained from untreated zygotes while the cytoplasm was from a zygote exposed to u.v. light under a fluorescence microscope after staining with Hoechst dye. No significant decrease of development to blastocysts was observed when the cytoplasm originated from eggs to u.v. light for 20–30 sec. However, the proportion of reconstituted eggs developed to blastocysts was significantly ($P < 0·01$) lower (23%) when the cytoplasm originated from eggs exposed for 40 sec compared with that observed when the cytoplasm was from control eggs (50%).

Table 2. The effect of exposure to u.v. radiation on in-vitro development of mouse zygotes pretreated with Hoechst 33342 dye

Conc. of dye (μg/ml)	Duration of u.v. exposure (sec)	No. of eggs used	No. of eggs developed to:	
			2-cell (%)	Blastocyst (%)
0	0	39	39 (100)	34 (87)
	5	30	29 (97)	26 (87)
	10	30	30 (100)	27 (90)
	20	30	29 (97)	24 (80)
	30	53	52 (98)	45 (85)
	40	53	53 (100)	43 (81)
	50	30	28 (93)	21 (70)
	60	53	51 (96)	45 (85)
	120	23	10 (43)**	7 (30)**
	240	25	5 (20)**	2 (8)**
	360	22	3 (14)**	1(5)**
0·5	0	30	30 (100)	30 (100)
	5	30	28 (93)	17 (57)**
	10	30	23 (77)*	14 (47)**
	20	30	19 (63)**	8 (27)**
	30	30	7 (23)**	0
1·0	0	30	30 (100)	26 (87)
	5	30	27 (90)	15 (43)**
	10	30	19 (63)**	7 (23)**
	20	30	11 (37)**	3 (10)**
	30	30	10 (33)**	0
2·5	0	30	30 (100)	27 (90)
	5	30	22 (73)*	4 (13)**
	10	30	14 (47)**	6 (20)**
	20	30	9 (30)**	0
	30	30	7 (23)**	0

*$P < 0{\cdot}01$; **$P < 0{\cdot}001$.

Table 3. Development of reconstituted eggs *in vitro* and *in vivo* with cytoplasm originated from zygotes exposed to u.v. for different periods after staining with Hoechst dye

Duration of u.v. exposure (sec)	No. of reconstituted eggs cultured	No. of eggs developed to blastocysts (%)	No. pregnant/ no. of recipients	No. of live fetuses/no. of blastocysts transferred (%)
0	64	32 (50)	4/4	5/12 (42)
20	48	18 (38)	3/4	7/16 (44)
30	54	22 (41)	3/4	7/17 (41)
40	47	11 (23)*	0/1	0/5 (0)

*$P < 0{\cdot}001$.

Series 2

The chromosomes at the second metaphase could easily be removed within 15 sec under a fluorescence microscope. Of 83 eggs receiving a single inner cell mass nucleus, 62 (75%) showed a pronuclear-like formation 6 h after nuclear transplantation and activation. Each nucleus had 1–8 nucleoli when it was examined under an inverted microscope (× 400). Of 62 eggs with a nucleus 14 (23%) developed to the 2-cell stage, with each blastomere having one nucleus, 1 day after activation. However, only 3 of them (5%) developed to the blastocyst stage after 4 days of incubation.

Discussion

The present study clearly demonstrated that the pronuclei of mouse zygotes are more sensitive to u.v. irradiation after Hoechst staining than is the cytoplasm. Although Hoechst dye is readily permeable and gives fluorescence to the chromosomes at metaphase, it will irreversibly modify nucleic acid and prevent normal configurational changes during mitosis and meiosis (Albertini, 1984). As indicated in Exp. 1 of Series 1, the treatment of zygotes with Hoechst 33342 at high concentrations (7·5 and 10 μg/ml) significantly inhibited the development to blastocysts.

Spielmann & Eibs (1978) and Eibs & Spielmann (1977) reported that irradiation with u.v. light having an output of 254 nm inhibited the developmental ability of mouse preimplantation embryos. They reported that the u.v. sensitivity was highest in zygotes, with a linear increase of abnormal eggs with u.v. dose. In our preliminary study using a u.v. lamp (254 nm), the proportion of mouse zygotes developing to blastocysts after irradiation with a dose of 1·4 erg/mm^2 was not different from that obtained in control eggs. After irradiation with a dose of 7 erg/mm^2, a significantly lower proportion of eggs (6/20, 30%) developed to blastocysts and most of them (7/209, 3%) did not develop to blastocysts when exposed to u.v. light at 14–168 erg/mm^2. The remaining eggs stopped developing at the 1-cell to morula stage. The damage caused by u.v. irradiation was also observed and was more serious in eggs examined under a fluorescence microscope after staining with Hoechst dye. The proportion of eggs developing to blastocysts after exposure for 5 sec under a fluorescence microscope was significantly lower than that obtained in control eggs. However, exposure to only u.v. light for 5–60 sec did not inhibit the development of eggs to blastocysts. As reported by Ebert *et al.* (1985), the adverse action of Hoechst staining and u.v. irradiation to mouse zygotes might be additive or even synergistic.

It is believed that damage caused by u.v. irradiation is mainly due to lesions of DNA in cells. The results in Exp. 3 of Series 1 showed that the pronuclei of mouse zygotes were more sensitive to u.v. irradiation after Hoechst staining than was the cytoplasm. The same situation was observed in our preliminary study when a u.v. lamp of wavelength 254 nm was used for irradiation without Hoechst staining. No significant decrease of developmental ability of reconstituted eggs to blastocysts (82/123, 67%) was observed when cytoplasm was from eggs irradiated at 168 erg/mm^2 with the u.v. lamp. After transfer of such blastocysts to recipients, the pregnancy rate (13/14, 93%) and the proportion of live fetuses (27/81, 33%) were not different from those which had been obtained after transfer of reconstituted eggs (Tsunoda *et al.*, 1986). However, no development was observed when the cytoplasm was from eggs irradiated with doses 15–30 times higher (0/17).

The results in Series 2 clearly demonstrate that the relatively low sensitivity of cytoplasm to u.v. exposure after Hoechst staining could be used for nuclear transplantation of inner cell mass nuclei to mouse eggs at second metaphase. McGrath & Solter (1984) reported that enucleated mouse zygotes which received nuclei from 4- and 8-cell embryos were unable to develop *in vitro*. Tsunoda *et al.* (1987) have reported that when the nuclei of 4- and 8-cell embryos were transferred to enucleated 2-cell embryos, they not only developed to the blastocyst stage, but also to full term. However, the nuclei transplanted into enucleated 2-cell embryos were not fully transformed to 2-cell nuclei, since most of these embryos compacted at the 4-cell rather than the 8-cell stage (Tsunoda *et al.*, 1987), and blastocysts developed from them were smaller and had fewer nuclei (Robl *et al.*, 1986). The reconstituted 2-cell embryos receiving inner cell mass nuclei did not develop at all (Tsunoda *et al.*, 1987). Orr *et al.* (1986) transplanted terminally differentiated frog erythrocytes into maturing oocytes at the first meiotic metaphase, activated the oocytes and then removed the second black dot indicating the recipient's meiotic metaphase. They obtained blastulae after such a nuclear transplantation and nuclear lines from erythrocyte nuclei after serial nuclear transplantations. There are technical difficulties in carrying out such procedures in the mouse. Since nuclei transplanted into oocytes at the first metaphase soon group around the recipient's chromosomes, it is impossible to distinguish the transplanted from the recipient nucleus (Y. Tsunoda, unpublished observation). However, when the nuclei were transplanted into eggs at second metaphase, most soon dispersed into the cytoplasm (Czołowska *et al.*, 1984). Czolowska *et al.* (1984) fused thymocyte nuclei with mouse

oocytes at the second metaphase by using polyethylene glycol. They observed that the thymocyte nuclei developed along a pronucleus-like pathway and increased up to 200 times in volume after activation. Since such eggs should have a diploid nucleus originating from the transplanted one, and a haploid or diploid nucleus of recipient origin, one could not expect normal development.

From the results in Series 2, we conclude that some inner cell mass nuclei transplanted into enucleated eggs at the second metaphase changed into 'fertilization' nuclei. It has been reported that the transit from maternal to embryonic control of development takes place at the 2-cell stage in the mouse (Johnson, 1981). The fact that only a few eggs receiving an inner cell mass nucleus developed beyond the 4-cell stage indicates that the conditions used in the present study were not adequate for conversion of the transplanted nucleus to a 'fertilization' one. It is also possible that the sensitivity of the cytoplasm at the second metaphase stage to u.v. irradiation and Hoechst staining may differ from that of cytoplasm at the pronuclear stage. Further studies, for example into the synchronization of cell cycle between cytoplasm and nucleus, and the optimal time between nuclear transplantation and activation, are required.

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